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ANALGESIC AND ANTI-INFLAMMATORY EFFECTS OF *NERVILIA PURPUREA* AND ITS ACTIVE COMPONENTS

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Nervilia purpurea (HAYATA) SCHLETHER (NP) (Orchidaceae), distributed in cool wet mountain areas of Taiwan, has been used as a folk medicine in Taiwan to treat the external injuries (such as bruises), weakness, pneumonia and hypertension. However, there are few reports about its pharmacological effects. In our preliminary study, the crude extract of NP has been observed to inhibit the writhing response markedly in mice induced by acetic acid. Thus, we separated the herb into two portions. Each extract was separated with various solvents to produce six layers that included some compounds. We attempt to explore the pharmacological properties of those active compounds. Following results were obtained: (1) the median lethal dose (LD50) of NP crude extract was higher than 15 gkg, orally. (2) the crude extracts of NP did not affect the nociceptive response in hot plate in mice. (3) substances in the ethyl acetate layer reduced acetic acid-induced writhing response. (4) the crude extract of NP could inhibit the inflammatory edema induced by carrageenin and formalin and the aqueous layer of leaf extract at most effective. (5) the ethyl acetate layer of leaf extract inhibited the skin permeability induced by PGE_2 in the skin windows test and attenuated the licking response in formalin test.

In conclusion, we suggest that NP possessed the analgesic and anti-inflammatory effects which may be related to the endogenous of prostaglandins.

Key Words : *Nervilia purpurea*, Analgesic, Anti-inflammatory effects

INTRODUCTION

Nervilia purpurea (HAY.) SCHLETHER (NP) is an herb of Orchidaceae. Its Chinese name contains the word "huang" (廣) which is understood to denote certain febrile or heat conditions[1]. It has been used as a folk medicine in Taiwan for treatment of injuries from knocks and falls (such as bruises), the "five taxations and seven damages" (五癆之傷) (various deficiencies or unhealthy conditions due to imbalance of activity and rest, or

emotional disturbance), pneumonia and hypertension. These indications suggest that it has anti-inflammatory and analgesic properties. Although some fatty acids and sterols were identified by Kikuchi et al[2-4].However, there are few reports about its pharmacolgical effects. Since our preliminary study showed the crude extract of NP had marked analgesic effects, we decided to study the pharmacological mechanisms in much detail and also to determine the constituents, with a view to developing medicinal applications.

MATERIALS AND METHODS

Ⅰ. Preparation of the materials

Firstly, 2.0 kg of the fresh herb was separated into leaves (o.55 kg) and tubers (1.45 kg) which were extracted with 50% ethanol. The extracts were concentrated in vacuum and dried to produce a 2.7% extract product. Extract of the leaves was 38.8g (1.94%), while that of the tubers, after removal of the starch, was 65.8g (3.29%). The extracts were dissolved in an appropriate quantity of water, and partitioned with ethyl acetate, and n-butanol etc. The leaf extract produced an ethyl acetate layer (L-EtOAc) of 8.6g (0.43%), a butanol layer (L-BuOH) of 4.0g (0.20%), and an aqueous layer (L-H_2O) of 20.0g (1.00%). The tuber extract produced an ethyl acetate layer (T-EtOAc) of 0.2g (0.01%), a butanol layer (T-BuOH) of 19.8g (0.99%), and an aqueous layer (T-H_2O) of 34.2g (1.71%). The leaf ethyl acetate layer was subjected to column chromatography. It was eluted with n-hexane and ethyl acetate in various proportions. Various compounds, including beta-sitosterol, stigmasterol, linolic acid, linolenic acid, palmitic acid, L-norleucine and phytol as well as NP-3 and NP-6 crystals were obtained by isolation and purification (Fig. 1).

Ⅱ. Acute toxicity

Using the Litchfield and Wilcoxon method[5], ICR strain male mice, weighing 18－25g, were administered orally the crude extract of the herb. They were monitored for poisoning and fatality for 72 hours. The LD50 and 95% confidence levels were established.

Ⅲ. Analgesic test

1. Acetic acid-induced writhing response test

The employed test was originally described by Taber et al[6]. Male ICR mice, weighing 18－25g , were randomly divided into several groups. Each mouse was injected intraperitoneally with 0.6% acetic acid in a volume of 0.1 ml/10 g of body weight. To each group (n＝8), normal saline (0.1 ml/10 g of body weight), aspirin (100 mg/kg), or NP extracts was administered intraperitoneally. Five minutes after the injection of acetic acid, the mice were observed for 10 minutes and the number of writhing response for each mouse was recorded.

The writhing reduction rate, or analgesic potency, was calculated.

2. Hot plate test

The method represented a modification of that described by Woolfe and MacDonald[7]. Male ICR mice, weighing 18－25 g, were used. The apparatus consisted of a metal plate maintained at temperature of 55±0.5°C by a hot plate analgesia meter (Muromachi Kikai MK-350). Mice were administered intraperitoneally 500 mg/kg of NP crude extracts. The reaction time was determined by the behavior of the animals, the end point being licking or kicking its hindpaw or jumping. It was measured and recorded at 40, 20 minutes before injection and at 0, 20, 40, 60 and 80 minutes following drug administration.

3. Formalin-induced licking response

The method represented a modification of that described by Dubuisson and Dennis[8]. Male ICR mice, weighing 18－25 g, were used. Prior to testing, the mice were allowed to the testing environment for at least 18 hours. Two hours before testing the animals were placed individually in standard cages which served as observation chambers. A mirror was placed behind the chamber. After an adaptation period, the mice were removed from the cage. NP, indomethacin or saline was respectively administered 0.5 hours before the formalin injection. Formalin solution (1% 20μl) was injected subcutaneously on the dotsal surface of the right hindpaw through a microsyringe with a 27－gauge needle. The mice were then returned to the chamber. The time that the mouse spent licking the injected paw was recorded, and was observed for 30 minutes.

Ⅳ. Anti-inflammatory test

1. Skin windows test

The vascular permeability evoked by the skin window model was performed according to the method described by Otani and Hugli[9]. Male Wistar rats, weighing 150－225 g, were used. Each rat was administered with the tested drugs intraperitoneally 30 minutes before the experiment. The rats were anesthetized with pentobarbital (30 mg/kg, ip). Their abdominal hair was shaved with an electric hair clipper. The rats were then given 2% Evans blue (1.5 ml/kg) into the penis vein. 50 ul each of inflammatory mediators platelet activating factor (PAF 40nM/ site), bradykinin (BK 10 nM/site), prostaglandin E_2 (PGE_2 2 nM/site), compound 48/80 (200 ng/site) or serotonin (5-HT 1 nM/site) was injected subcutaneously in each of abdominal centralateral regions. The rat was sacrificed 30 minutes after injection of the mediators. A 20 mm diameter piece of blue area skin was removed from the experimental sites. It was chopped into small pieces and placed in a tube containing a mixture of 0.5% aqueous sodium sulfate (3 ml) and acetone (7 ml). After 24 hours, the tissues were removed from the reagent. The suspension was centrifuged at 4°C (600 g for 20 minutes). The

concentration of dye in the tube was measured by spectrophotometer (Bacharach coleman, model 35) at 620 nm.

2. Hindpaw edema test

The vascular permeability of hindpaw edema model was performed according to the method described by Winter[10]. Male Wistar rats, weighing 180−225 g, were randomly allocated. NP, indomethacin or saline was administered 0.5 h before the induction of edema. Paw edema was induced by injection of 50 ul 1% lambda-carrageenin, 0.1% serotonin, or 1% formalin into the subplantar of rat hindpaw. Hindpaw volume was measured by a water plethysmometer (Ugo Basile, model 7150) before injection and at 30 minutes intervals after injection of the irritant , and continued for more than 5 hours. The increase of percentage of volume was calculated according to the following equation.

$$\text{Edema rate (E1)} = \frac{B1 - A}{A} \times 100 \text{ (the first time)}$$

A1: lst control volume of displacement

B1: lst volume of displacement after injection

B2: 2nd volume of displacement after injection

V. Statistical analysis of results

Results were expressed as mean±S.E. The unpaired Student's t−test or paired Student's t−test were used for statistical evaluation of the results. Statistical significance was set at $P < 0.05$.

RESULTS

Ⅰ. Acute toxicity

The LD50 of crude NP extract administered orally to ICR strain male mice was more than 15 g/kg. The drug showed a high of safety.

Ⅱ. Analgesic test

1. Acetic acid-induced writhing response

Crude extracts of the whole herb, leaf, or tuber was administered intraperitoneally in mice in doses of 1.0 g/kg. All exhibited analgesic effects as compared with 100 mg/kg aspirin (Fig. 2A). Among the layer, the leaf ethyl acetate layer showed the most effective (Fig. 2B). This layer was administered intraperitoneally in doses of 25, 50, and 100 mg/kg, and the analgesic effect was dose-dependent (Fig. 3).

2. Hot plate test

NP administered intraperitoneally in doses of 500 mg/kg did not prolong the reaction time to pain induced by conductive heat.

3. Formalin-induced licking response in mice

Crude extracts of the whole herb, leaf and tuber administered intraperitoneally had little inhibitory on the early-phase of formalin-induced licking response (Fig. 4A). However, they showed marked inhibition in the late phase. The crude extract of leaf showed the strongest effect (Fig. 4B). Further screening showed that the various layers of the leaf and tuber extract exerted no inhibition as compared with the control group (data not shown). The ethyl acetate layer of the leaf extract had a potential inhibitory effect on late phase licking time and the n-butanol layer a less potent inhibition (Fig. 5). This inhibition was dose-dependence.

The ethyl acetate layer of leaf was eluted by column chromatography with hexane and ethyl acetate. The eluates were then purified by chromatography and/or recrystalization. Several compounds including beta-sitosterol, stigmasterol, linolic acid, linolenic acid, palmitic acid, L-norleucine and phytol were obtained. These seven compounds produced no significant inhibition on the early phase of licking response (data not shown). However, beta-sitosterol, palmitic acid, stigmasterol and L-norleucine did show an inhibition on the late phase (Fig. 6). Butanol and the water insoluble part of the ethyl acetate layer yielded NP-3 and NP-6 crystals which proved to have no significant effect on either early or late phase of the licking response.

Ⅲ. Anti-inflammatory test

1. Skin windows test

The leaf ethyl acetate layer administered intraperitoneally had a marked inhibition on excessive permeability in cutaneous vessels induced by PGE_2 (2 nM/site). The tuber aqueous layer showed a marked inhibition on excessive permeability induced by PGE_2 and PAF. The effect was dose-dependent. Neither layer had marked effect in excessive permeability in the cutaneous vessels induced by bradykinin (10 mM/site) and compound 48/80 (200 ng/site) (see Fig. 7).

2. Hindpaw edema test

The crude extracts of NP administered intraperitoneally showed a mild inhibition on serotonin-induced hindpaw edema (Fig. 8A), and had significant effect on formalin-induced edema at doses of 1.0 g/kg (Fig. 8B). However, it showed a dose-dependent inhibitory effect on the hindpaw edema induced by 1% lambda-carrageenin (Fig. 8C). The inhibition of leaf aqueous layer on the lambda-carrageenin induced edema was marked. It also showed a dose-dependent action (Fig. 8D).

DISCUSSION

Randall [11] ,Siegmund [12],Collier [13],and Teber [14] reported that chemical substances could induce nociceptive response in animals. Intraperitoneal injection of acetic acid produced an abdominal writhing response by activating the chemosensitive nociceptors in animals [6]. Narcotic drug such as opioids, endogenous opioid peptides, steroid and nonsteroid anti-inflammatory agents could inhibit the writhing response, and provided an experimental model for the screening of analgesics[11]. The present experiment showed that the ethyl acetate layer of NP significantly inhibited the acetic acid-induced writhing response in mice. However, the acetic acid-induced writhing response could not determine whether the analgesic effect was due to the central inhibition or the peripheral nociceptive mediators, further analysis was required.

NP crude extract administered intraperitoneally at doses of 500 mg/kg, had on effect in the thermally induced nociceptive responses. According to Woolfe [7], narcotic agents, but not aminopyrine or aspirin, could inhibit these responses, and represented central analgesic actions. It implied that NP was not a central acting analgesics.

The formalin-induced licking response was an ideal model for evaluating new analgesics [15–19]. Mice were administered 1% formalin 20 ul intradermally on the dorsal aspect in the mouse hindpaw. The period of this nociceptive responses induced by formalin might be divided into two phases, the early phase from 0 to 5 minutes, and the late phase from 15 to 30 minutes. The pain response of the early phase was related to the conduction of nociception in peripheral nerve ending and the release of neurotransmitters (including substance P etc.). The late phase was a secondary reaction to cell damage that promoted the production of histamine, serotonin, prostaglandins, and bradykinin [16–20]. NP produced less inhibition on the formalin-induced licking response in the early phase, which further showed that it did not relieve pain by acting on the central nervous system. However, the crude extracts of both leaf and tube affected the licking time. The leaf ethyl acetate layer was markedly effective. Further analysis demonstrated that beta-sitosterol, palmitic acid and L-norleucine inhibited the later phase licking. We confirmed the anti-inflammatory and antipyretic effects of beta-sitosterol reported by Gupta [21].

The lambda-carrageenin-increased vascular permeability in inflammatory conditions in rats were usually biphase responses. In the initial stage it was related to the release of histamine and related substances [22]. In the late stage, however, it was associated with plasma kinins and tissue prostaglandins. Rebuck [23] reported that vascular permeability could be

detected by the skin window test. Inflammatory chemical mediators such as histamine, bradykinin, PGE_2, PAF and compound 48/80 were used to induce vascular permeability. Histamine, bradykinin, and PAF were known for their potency in increasing vascular permeability[24–30]. PGE_2 could enhance the vascular permeability of bradykinin as a potentiator. Compound 48/48 was known to degranulate mast cells and induce the release of histamine and serotonin which increased the vascular permeability [23]. The present study showed that the leaf ethyl acetate layer and the tuber water layer of NP had an inhibition on excessive vessel permeability caused by PGE_2. The tuber extract inhibited the excessive permeability caused by PAF. None of the layers of NP had any inhibition on excessive vascular permeability induced by compound 48/48, bradykinin or histamine. These findings show that the anti-inflammatory effect of the leaf ethyl acetate layer was intimately related to PGE_2. The leaf water layer was related to PAF rather than bradykinins, serotonin or histamine.

Serotonin was a potential microvascular dilator that directly increased vascular permeability and created local edema in rats. Large doses of NP had no effect on hindpaw edema induced by serotonin. In addition to causing pain by directly prompting a neural signal, formalin could also cause regional edema when it is injected into the rat hindpaw. It causes cell damage by inducing the production of the endogenous mediators, histamine, serotonin, prostaglandins or bradykinin [15]. This reaction was significantly inhibited by large doses of NP. Carrageenin-induced hindpaw edema had a pronounced biphasic course with different inflammatory chemical mediators being released at different times. At the first 90 minutes period following injection of carrageenin, histamine and serotonin were released. While during the period from 90 to 150 minutes affter injection of carrageenin, kinins were released and affer 150 minutes prostaglandins and leukotrienes were produced [31–37]. Our experiment showed that the leaf aqueous layer of NP had marked inhibition on the carrageenin-induced edema. Ninety minutes after the injection, it produced the effect and lasted for over five hours. From the hindpaw edema test, it is evident that the leaf aqueous layer had an anti-infl-ammatory effect which was closely related to the inflammatory chemical mediators of prostaglandin or leukotrienes, but bearing little relation to histamine and bradykinin.

From these findings, it is concluded that NP crude extract had no effect on thermal-induced nociceptive response. The analgesic mechanism of NP appeared not to act on the central nervous system. The lesser effect on the early phase of formalin-induced licking response further proved that it did not on the central nervous system. The increasing vascular permeability test showed that the ethyl acetate layer and the aqueous layer of the leaf of NP

had marked inhibition on excessive vascular permeability caused by PGE_2. However, the tuber aqueous layer inhibited the excessive permeability caused by PAF. In the paw edema test, NP had no inhibitory effect on serotonin hindpaw edema. In the formalin edema test, it was found to be significantly inhibited only at large doses. However, the leaf aqueous layer had marked effect on carrageenin-induced edema. From the above results, we suggest that the analgesic and anti-inflammatory effects of *Nervilia purpurea* might be related to the inhibition of prostaglandins. Whether it had the other mechanisms deserves further studies.

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LEGEND

Fig. 1 Extraction scheme of *Nervilia purpurea*.

Fig. 2 Analgesic effect of *Nervilia purpurea*(NP) on acetic acid-induced writhing response in mice. A.– –Crude extracts of herb, leaf and tuber (100 mg/kg, ip). B.– –Individual layer of leaf (L) and tuber (T) of NP. Agents were administered intraperitoneally at 100 mg/kg 30 minutes before acetic acid injection(n=8).

Fig. 3 Dose-response curve of leaf ethyl acetate layer on acetic acid-induced writhing response in mice. Inhibition of writhing response is shown as the percentage in comparison to the control group value. Each point represents the mean±S.E.(n=8).

Fig. 4 Antinociceptive effects of NP extracts administered intraperitoneally in the formalin test in mice. A.– –Nociceptive response in the early phase (0–5 minutes after formalin injection). B. – – Nociceptive response in the late phase (15–30 minutes after formalin injection). Significant differences between saline and test drug groups are indicated by *$p<0.05$, **$p<0.01$, and***$p<0.001$.

Fig. 5 Antinociceptive effects of intraperitoneal administration of each fraction of leaf and tuber of NP in the late phase of the formalin test. Significant differences between saline and test drug groups are indicated by *$p<0.05$, **$p<0.01$, and ***$p<0.001$.

Fig. 6 Antinociceptive effects of seven compounds of leaf ethyl acetate layer administered intraperitoneally at 100 mg/kg in late phase of the formalin test in mice. Each bar represents the mean±S.E. (n=8). Significant differences between saline and test groups are indicated by *$p<0.05$, **$p<0.01$, and ***$p<0.001$.

Fig. 7 The effect of leaf ethyl acetate layer (L.E.) and tuber aqueous layer (T.H.) of NP in skin window test. The plasma extravasation response caused by a. bradykinin (BK 10 nM/site), b. prostaglandin E_2 (PGE_2 2 nM/site), c. serotonin (5–HT, 1 nM/site) d. compound 48/80 (200 ng/site), e. platelet-activating factor (PAF 40 nM/site) in rats. Histograms represent the mean±S.E. (N=8). Asterisks represent significant levels compared with saline the control group. *$p<0.05$, **$p<0.01$, and ***$p<0.001$.

Fig. 8 Inhibition of hindpaw edema by NP. Inflammatory mediator-induced edema by formalin (Fig. 8 A), serotonin (Fig. 8B), and carrageenin (Fig. 8C) in rats (n=7). The leaf aqueous layer (L.H.) showed a marked inhibition in carrageenin-induced edema, and had a dose-dependent effect (Fig. 8D). Each point represents increase ratio (%) of the mean of volume. Asterisks represent significant levels by Student's t–test. *$p<0.05$, **$p<0.01$, ***$p<0.001$, as compared with the saline control group.

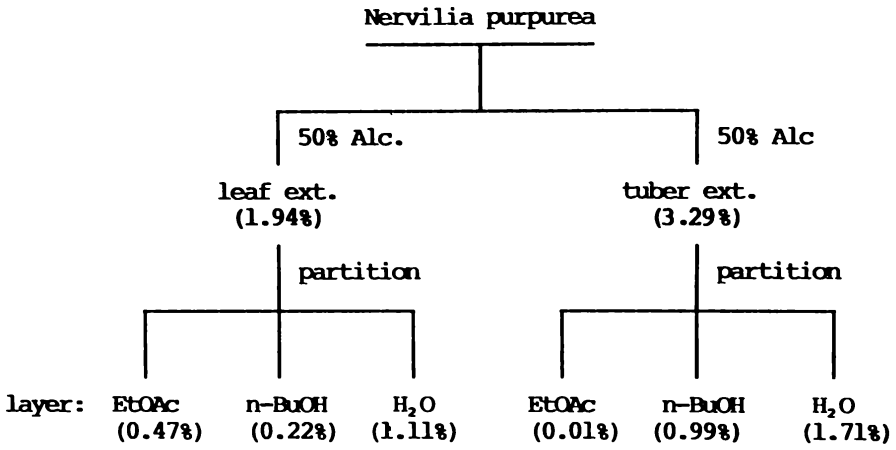

Figure 1

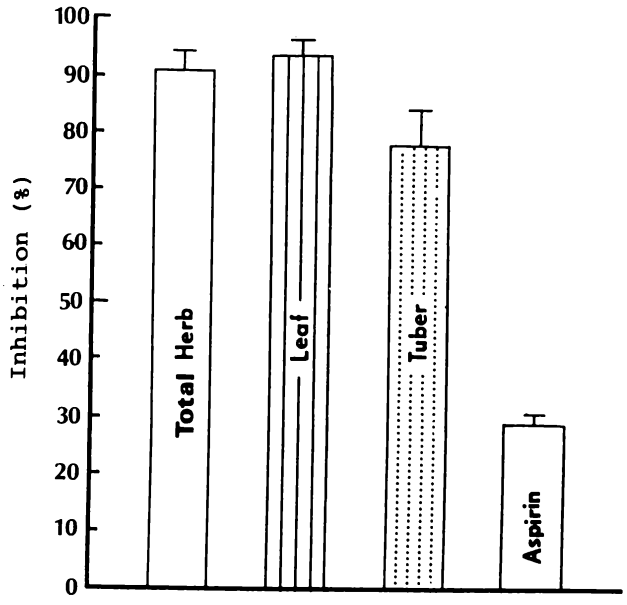

Figure 2A

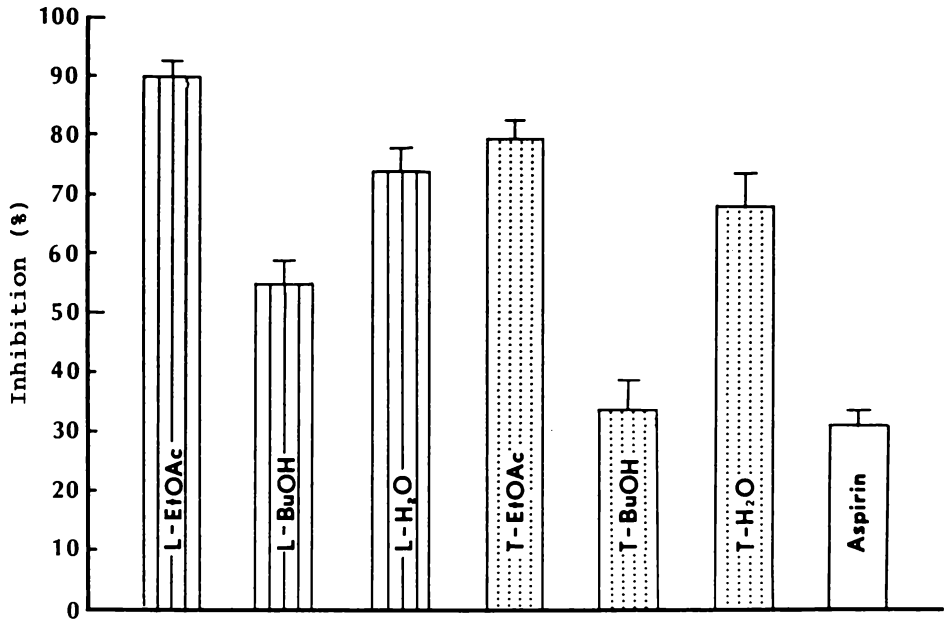

Figure 2B

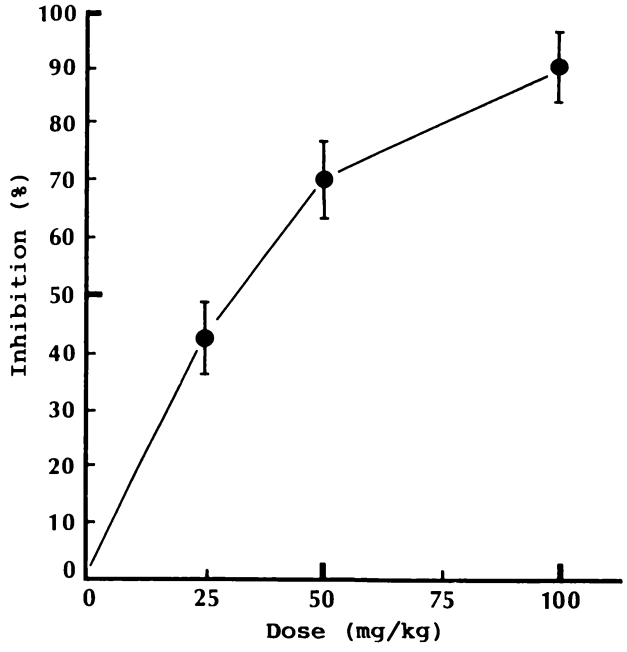

Figure 3

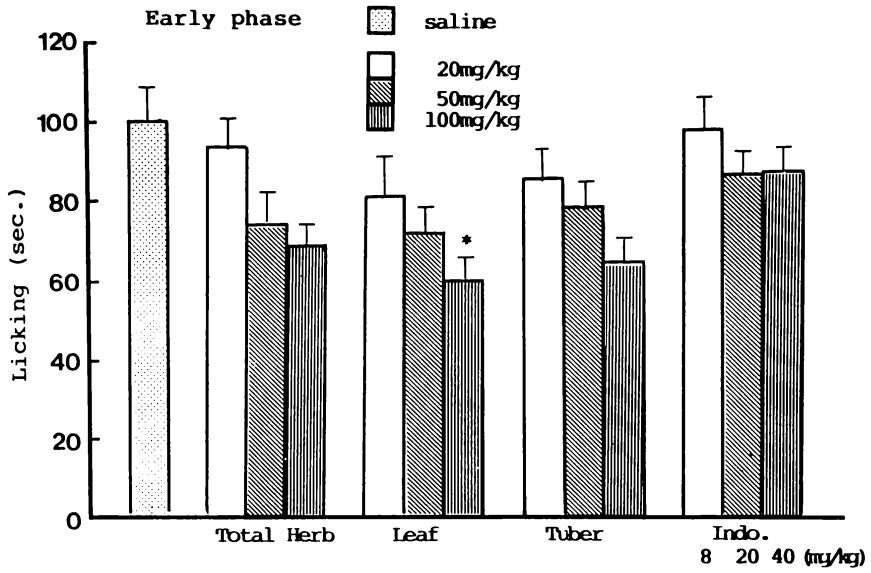

Figure 4A

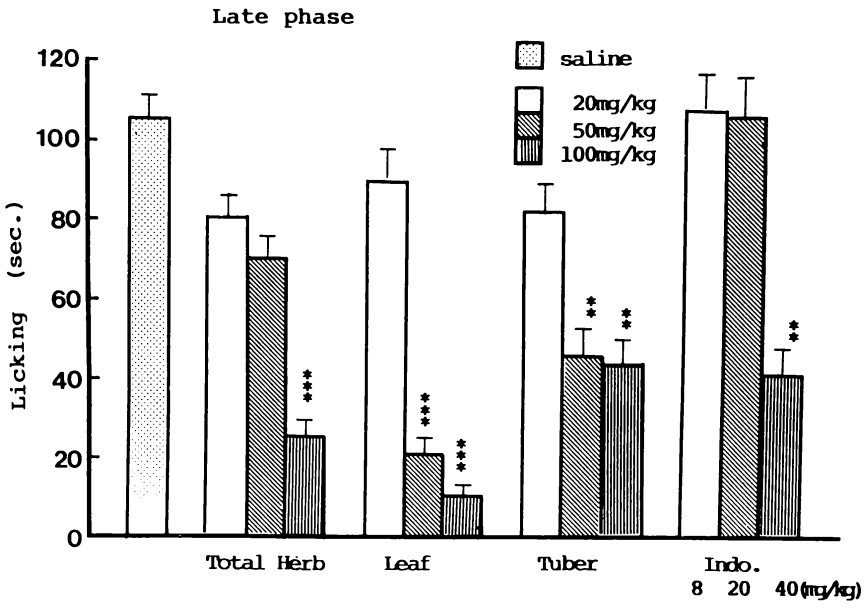

Figure 4B

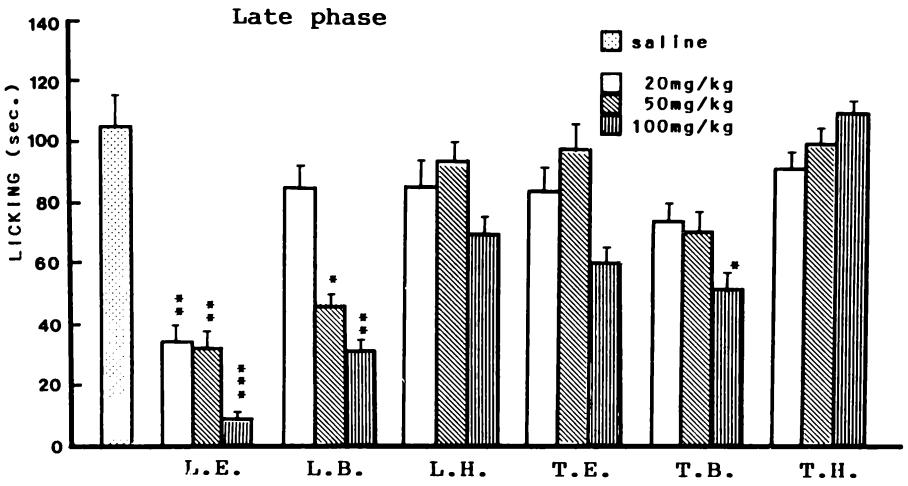

Figure 5

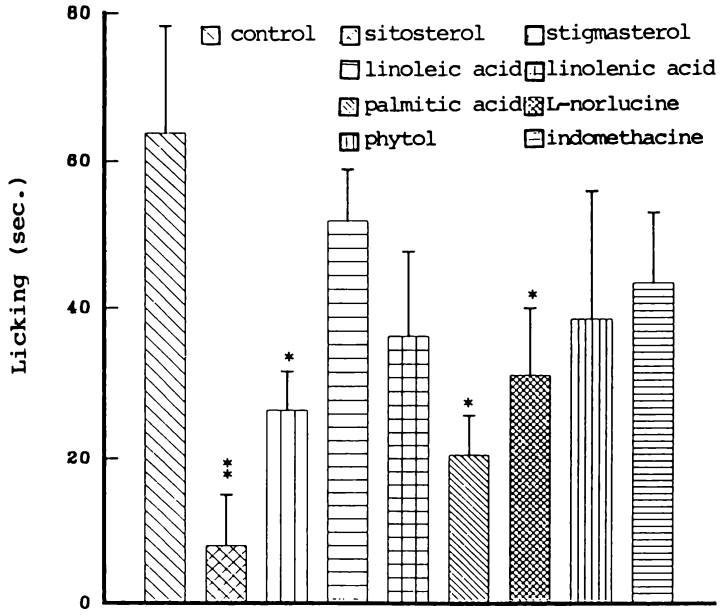

Figure 6

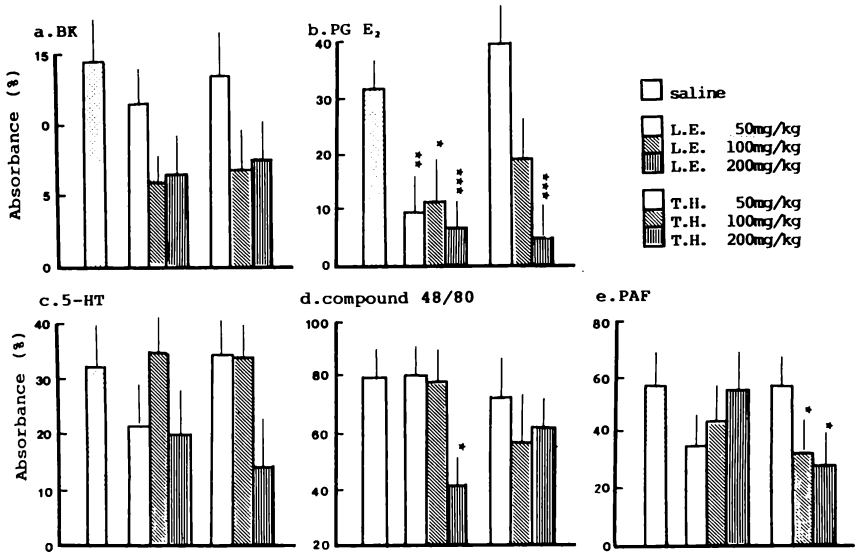

Figure 7

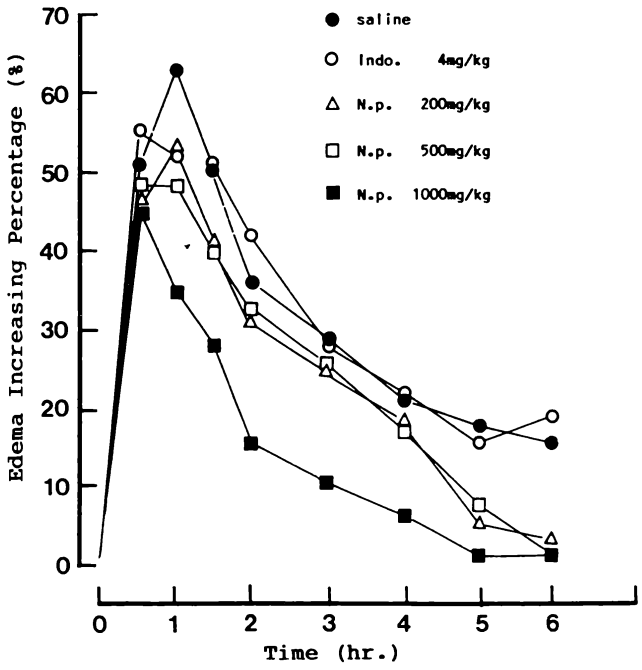

Figure 8A

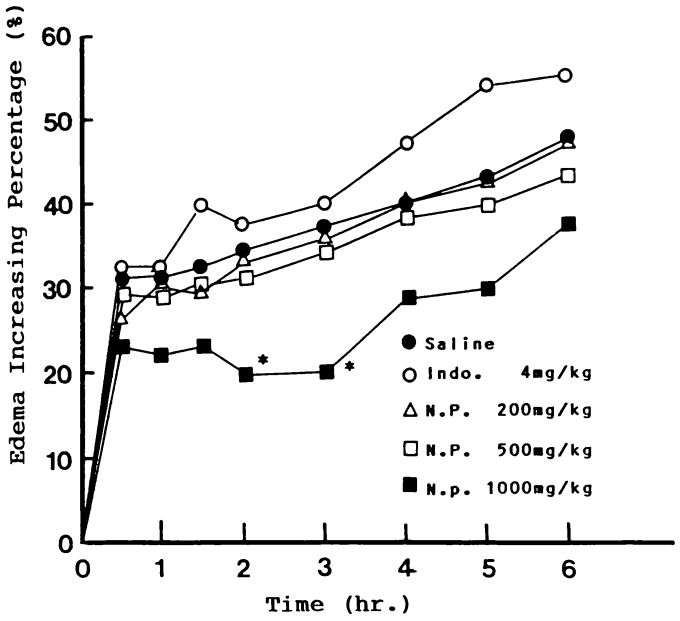

Figure 8B

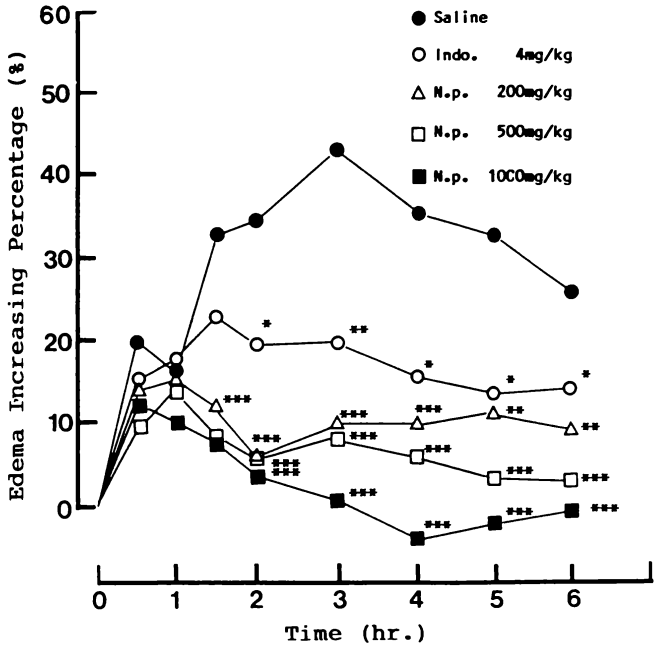

Figure 8C

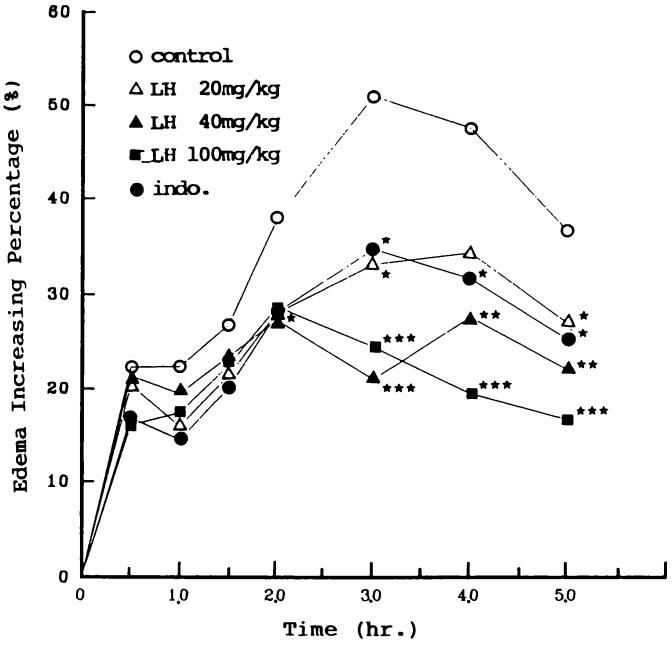
control
LH 20mg/kg
LH 40mg/kg
LH 100mg/kg
indo.
Edema Increasing Percentage (%)
Time (hr.)
0
10
20
30
40
50
60
1.0
2.0
3.0
4.0
5.0

Figure 8D

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紫背一點癀鎮痛抗炎作用及其有效成分之研究

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紫背一點癀(*Nervilia purpurea*)(Hayata)(Schlether)，係爲蘭科植物，生產於臺灣中海拔陰濕的山區，依據文獻記載民間嘗用於抗炎、退癀及治療高血壓，但目前尚無其藥理研究報導。本研究室經初步篩選的試驗，發現紫背一點癀全草50％酒精／水抽出物，具有抑制小白鼠對醋酸所誘發的扭體反應。故依其形態將紫背一點癀分成葉及球莖兩部分，分別先以50％酒精水溶液加熱抽取，抽取液再用溶媒進行分配分離，各得乙酸乙酯層、正丁醇層及水溶等層。葉之乙酸乙酯層經柱層層析並經純化後得數個化合物，經進行藥理試驗，獲致結果如下：

一、紫背一點癀全草粗抽出物對於ICR系雄性小鼠口服投與，劑量高達15g／kg，仍未發現動物死亡的現象，其毒性顯現很低。二、紫背一點癀500mg／kg對小鼠熱板誘發之痛覺無明顯抑制作用。三、葉之乙酸乙酯層對小鼠以醋所誘發的扭體反應抑制作用較大。四、紫背一點癀於carrageenin及formalin等誘發大白鼠後足蹠浮腫試驗，具明顯有抑制作用。其抗發炎作用，以葉之水層最強。五、葉之乙酸乙酯層以大白鼠皮膚視窗法進行試驗，發現能抑制PGE_2所誘發的滲透性增加作用。而且於對小鼠福馬林誘發的舔反應抑制作用較大。

根據實驗結果，紫背一點癀之鎮痛抗炎作用，可能是藉由抑制PGE_2等所產生的結果，其機理正進一步探討中。

關鍵詞：紫背一點癀，鎮痛，抗炎作用